Sex pheromone biosynthetic pathway for disparlure in the gypsy moth, *Lymantria dispar*

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The pheromone biosynthetic pathway for production of the sex pheromone disparlure, 2-methyl-7*R*,8*S*-epoxy-octadecane, was determined for the gypsy moth. Each step in the pathway was followed by using deuterium-labeled compounds that could be identified by using GC/MS. This approach provides unequivocal determination of specific reactions in the pathway. It was shown that the alkene precursor, 2-methyl-*Z*7-octadecene, is most likely made in oenocyte cells associated with abdominal epidermal cells. The pathway begins with valine contributing carbons for chain initiation, including the methyl-branched carbon, followed by chain elongation to 19 carbons. The double bond is introduced with an unusual Δ12 desaturase that utilizes a methyl-branched substrate. The resulting 18-methyl-*Z*12-nonadecenoate is decarboxylated to the hydrocarbon, 2-methyl-*Z*7-octadecene. The alkene is then transported to the pheromone gland through the hemolymph, most probably by lipophorin. At the pheromone gland, the alkene is unloaded and transformed into the epoxide disparlure for release into the environment. A chiral HPLC column was used to demonstrate that the (*R*,*S*)-stereoisomer of the epoxide, (+)-disparlure is found in pheromone glands.

Many female moths produce a sex pheromone for attraction of a conspecific mate. Sex pheromones from about 1,600 species of female moths have been identified (H. Arn, www.nysaes.cornell.edu/pheronet/ or www-pherolist.slu.se/). Most structures are fairly simple, consisting of 8–21 carbons in a chain with a functional group of either an alcohol, aldehyde, or acetate ester and usually one or more double bonds. In addition, some moths use hydrocarbons or epoxides of hydrocarbons as a sex pheromone.

The structure of sex pheromones indicates they are biosynthesized through modifications of fatty acid biosynthetic pathways. The key enzymes involved include desaturases, chain-shortening enzymes, reductases, oxidases, and acetyltransferases (1–3). A variety of desaturases have been identified that introduce the double bond, and these, along with chain-shortening enzymes, produce the large number of fatty acid intermediates that can be converted to sex pheromones (4). The biosynthesis of oxygenated pheromones occurs in pheromone gland cells. In contrast, little is known about the biosynthesis of hydrocarbon or epoxide sex pheromones.

The gypsy moth is found in Europe and Asia and as an introduced species in the northeastern U.S. Its larvae are serious defoliators of deciduous trees. The adult female does not normally fly and uses a sex pheromone to attract mates from long distances. The sex pheromone 2-methyl-7*R*,8*S*-epoxy-octadecane (disparlure) was identified (5) and is used in monitoring programs (6) and disruption treatments (7). On the basis of electroantennogram and behavioral assays, it is thought that female gypsy moths are producing only the (+)-stereoisomer of disparlure (8). The (–)-stereoisomer can be detected by the male moth but results in an inhibitory response (9, 10). In the present study, we were interested in determining how the female moth produces disparlure and in using chiral chromatography to determine its stereoisomeric composition. We determined that the biosynthetic pathway originates in oenocyte cells that produce the alkene, 2-methyl-Z7-octadecene (2me-7-18:Hc), which is then transported to the pheromone gland where it is converted to the epoxide disparlure with the majority being the *R*,*S* (+)-stereoisomer.

Materials and Methods

Insects. Gypsy moths were provided by the gypsy moth-rearing unit at the Otis Plant Protection Center, U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Plant Protection and Quarantine (Otis Air National Guard Base, MA). Female pupae were shipped by overnight express to Iowa State University, where they were allowed to emerge under a 14:10-h light/dark photoperiod. One- to 3-day-old females were used throughout this study.

Compounds. Deuterium-labeled valine (D_8-valine) was purchased from C/D/N Isotopes (Quebec). The (+)- and (–)-disparlure samples were a kind gift of J. Oliver (Agricultural Research Service, U.S. Department of Agriculture) and were >98% pure. All other reagents were purchased from Sigma.

The deuterium-labeled compounds D_4-2me-7-18:Hc, D_4-18-methyl-nonadecanoic acid (D_4-18me-19:COOH), and D_4-18-methyl-Z12-nonadecenoic acid (D_4-18me-12-19:COOH) were made as shown in Fig. 1. The deuterium label was introduced by deuteration of protected propargyl alcohol **3**. The protecting group was hydrolyzed, and the resulting alcohol was converted into the bromide **6**, which was transformed into the corresponding phosphonium salt. A Wittig reaction with the suitable aldehyde furnished the labeled intermediate **7**, which was hydrolyzed and further oxidized to produce the labeled acid D_4-18me-12-19:COOH. Hydrogenation of **7** and further hydrolysis and oxidation gave the saturated acid D_4-18me-19:COOH. Reaction of undecanal with the phosphorane obtained from the deuterated bromide **6** afforded the alkene D_4-2me-7-18:Hc. The acids were purified (>99%) by flash chromatography on silica gel by using 15% ethyl acetate/hexane as eluent. D_4-2me-7-18:Hc was purified by flash chromatography on silica gel by using hexane as eluent to obtain a 95/5 mixture of *Z*/*E* isomers. Repurification by flash chromatography on silica gel impregnated with $AgNO_3$ (10%) using hexane as eluent afforded pure (*Z*)-alkene.

(*Z*)-[14,14,15,15-$^2H_4$]-18-Methyl-12-nonadecenoic acid (D_4-18me-12-19:COOH). IR 2,925, 2,855, 1,710, 1,470, 1,410 cm^{-1}; ^1H NMR δ 5.37 (m, 1H), 5.35 (t, J = 4.5 Hz, 1H), 2.35 (t, J = 7 Hz, 2H), 2.01 (m, 2H), 1.62 (m, 2H), 1.52 (sept, J = 6.5 Hz, 1H), 1.44–1.21 (20H), 1.17 (m, 2H), 0.86 (d, J = 6.5 Hz, 6H); ^{13}C NMR δ 179.6, 129.9, 129.8, 38.9, 34.0, 29.8, 29.6, 29.5, 29.4, 29.3, 29.3,

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Abbreviations: 2me-7-18:Hc, 2-methyl-Z7-octadecene; 18me-19:COOH, 18-methyl-nonadecanoic acid; 18me-12-19:COOH, 18-methyl-Z12-nonadecenoic acid; D_8-valine, deuterium-labeled valine; SIM, single-ion monitoring.

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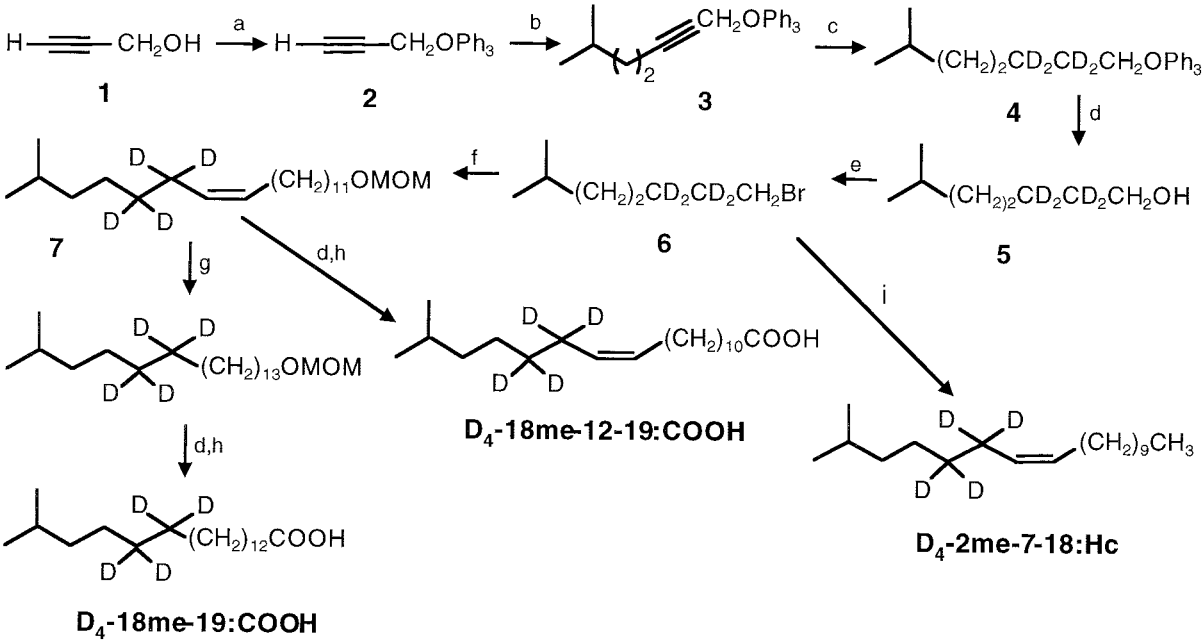

Fig. 1. Synthesis of labeled compounds. Reagents and conditions: (*a*) Trityl chloride/pyridine/25°C/24 h (95%); (*b*) *n*-BuLi/THF/0°C, then 1-iodo-3-methylbutane/HMPA/0–25°C/2 h (92%); (*c*) D_2/RhCl(PPh_3)$_3$/benzene/25°C/36 h (99%); (*d*) MeOH/$CHCl_2COOH$/25°C/48 h (90%); (*e*) NBS/DMF/Ph_3P/25°C/1 h, then MeOH/25°C/5 min (75%); (*f*) 1, Ph_3P/CH_3CN/reflux/24 h; 2, *n*-BuLi/THF/HMPA/–50°C/30 min, then 13,15-dioxahexadecanal/THF/–78°C/1 h, then 25°C/3 h (60%); (*g*) H_2/Pd/CmeOH/25°C/48 h (90%); (*h*) PDC/DMF/25°C/48 h (70–75%); (*i*) 1, Ph_3P/CH_3CN/reflux/24 h; 2, *n*-BuLi/THF/HMPA/–50°C/30 min, then undecanal/THF/–78°C/1 h, then 25°C/3 h (69%).

29.1, 28.0, 27.2, 26.8, 24.7, 22.6. Anal. calcd for $C_{20}H_{34}{}^2H_4O_2$: C, 76.37; H, 12.18. Found: C, 76.23; H, 12.03.

[14,14,15,15-$^2H_4$]-18-Methyl-nonadecanoic acid (D_4-18me-19:COOH). mp 68–71°C; IR 2,920, 2,850, 1,700, 1,470, 1,410 cm^{-1}; ^1H NMR δ 2.35 (t, J = 7.5 Hz, 2H), 1.63 (m, 2H), 1.51 (sept, J = 6.5 Hz, 1H), 1.42–1.20 (24H), 1.15 (m, 2H), 0.86 (d, J = 6.5 Hz, 6H); ^{13}C NMR δ 179.5, 39.0, 33.9, 29.7, 29.6, 29.4, 29.2, 29.1, 28.0, 27.2, 24.7, 22.7. Anal. calcd for $C_{20}H_{36}{}^2H_4O_2$: C, 75.88; H, 12.74. Found: C, 75.84; H, 12.64.

(*Z*)-[5,5,6,6-$^2H_4$]-2-Methyl-7-octadecene (D_4-2me-7-18:Hc). IR 2,955, 2,925, 2,855, 1,465 cm^{-1}; ^1H NMR δ 5.41–5.29 (2H), 2.02 (m, 2H), 1.52 (sept., J = 6.5 Hz, 1H), 1.44–1.21 (16H), 1.17 (m, 2H), 0.86 (d, J = 6.5 Hz, 6H); ^{13}C NMR δ 129.9, 129.8, 38.9, 31.9, 29.8, 29.7, 29.6, 29.4, 29.3, 28.0, 27.2, 26.8, 22.7, 22.6, 14.1 MS m/z 270 (M^+, 2), 127 (8), 113 (12), 97 (23), 85 (30), 69 (50), 57 (100). Anal. calcd for $C_{19}H_{34}{}^2H_4$: C, 84.35; H, 14.17 Found: C, 84.29; H, 14.19

Incubations. Labeled compounds were injected into intact females or incubated with isolated tissues. Injections were performed starting with 1-day-old females and were injected once daily for 3 days. Two hours after the last injection, hemolymph and pheromone glands were removed as described (11) and analyzed for incorporation of labeled precursors as described below. Incubation with isolated tissues consisted of first removing the majority of the scales on the abdomen by application of a gentle vacuum and then removing the abdomen at the junction of the thorax. The abdomen was cut laterally and pinned open. The ovaries were removed along with the majority of the remaining fat body, muscle, heart, and trachea, leaving mostly epidermal tissue attached to the cuticle. Tissues that were incubated include fat body, ovaries, and epidermal cells attached to the cuticle. These were incubated with labeled precursors in Grace's medium (GIBCO), as indicated in individual experiments. The deuterium-labeled fatty acids were transferred to a vial, the solvent was evaporated, and Grace's medium was added. The vial was sonicated in a water bath for 5 min before incubation with tissues. For isolated cuticular abdominal tissue, most internal tissue was removed and the abdomen was left pinned open. Grace's medium with or without labeled compounds was added to the cellular side of the abdomen and kept in a humidified chamber at room temperature. After various incubation times, an equal volume of methanol was added to the tissue and disrupted with a probe sonicator. Hexane was then added and vortex mixed, and the hexane layer was removed for analysis. Isolated pheromone glands (12) were incubated by floating on 10 μl of Grace's medium with or without D_4-2me-7-18:Hc. After the indicated incubation time, the gland was removed and extracted in hexane.

GC/MS Analysis. Label incorporation was determined by GC/MS. A Hewlett–Packard 5972 mass selective detector was coupled to a Hewlett–Packard 5890 GC equipped with a DB-5 (J & W Scientific, Folsom, CA) capillary column (30 m $\times$ 0.25 mm). The oven was temperature programmed starting at 80°C for 1 min, then increased at 10°C per min to 320°C. The MS was set in either the scan or single-ion monitoring (SIM) mode. When the SIM mode was used, specific ions were identified based on characteristic ions for each compound of interest, and the deuterium label was followed by adding the number of deuterium ions present in the precursor. Dimethyl disulfide derivatives were made as described (13).

Hemolymph samples were collected and extracted and hydrocarbons were purified as described (11). Pheromone glands were extracted in hexane and the hexane extract was analyzed directly by GC/MS. Isolated tissues were extracted first in methanol, then hexane, and the hydrocarbons in the hexane were purified as described (11).

HPLC/MS Analysis. The stereoisomers of disparlure were separated by chiral HPLC by using a Chiralcel OJ-R (Daicel Chemical, Tokyo) column (2.1 $\times$ 150 mm) in a Hewlett–Packard 1090

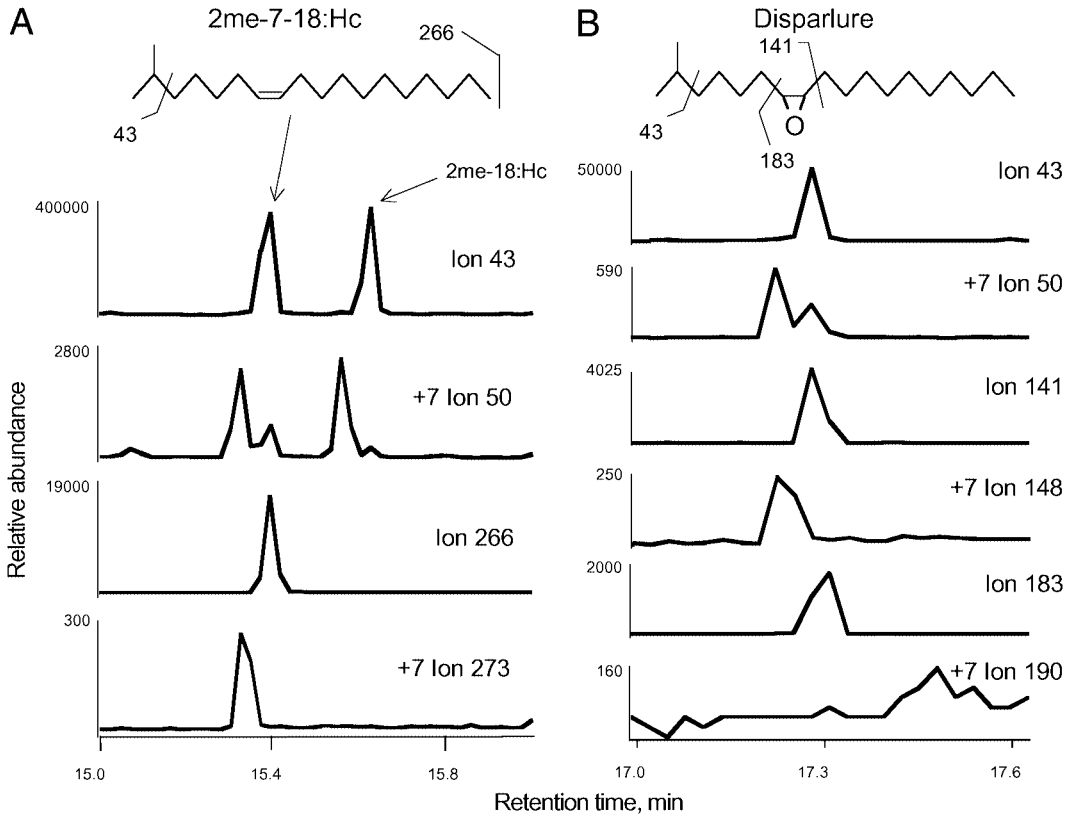

Fig. 2. Valine incorporation into the alkene 2me-7-18:Hc (*A*) and disparlure (*B*). D_8-Valine (20 μg per 20 μl of saline) was injected into 1-day-old females once daily until 3 days old (three injections total). One hour after the last injection, hemolymph and pheromone glands were extracted and hydrocarbons were purified and analyzed by GC/MS. MS analysis was performed in the SIM mode, analyzing for specific ions plus 7, because one deuterium is lost during the conversion of valine to isobutyryl-CoA. The ions that were monitored were 43 and 266 for the alkene. These were selected because 266 is the molecular ion and 43 can come from the methyl branch and both are of significant intensity. The ions 43, 141, and 183 were monitored for disparlure. These ions were selected because of their intensity, and the latter two are fragments on either side of the epoxide. Control females were injected with unlabeled valine, and the peaks for ions 50 and 273 were absent in the alkene (data not shown). The labeled ion peaks elute just before the natural unlabeled peaks, which is indicative of deuterium labeling.

HPLC coupled to a 1100 mass selective detector. An isocratic flow of 200 μl/min of methanol/water (85:15) was used for elution. The mass selective detector was used at positive polarity, atmospheric pressure ionization–electrospray (API-ES) ionization mode, and the fragmentor set at 70. SIM was used, detecting ions 283 (M + 1) and 300 (M + 18). The M + 18 ion was monitored due to the addition of ammonia, because ammonium acetate was present in the mass selective detector. Pheromone glands were removed from 2-day-old females and extracted in hexane. Disparlure was purified from the hexane extract by using a column of silica as described (11).

Results and Discussion

The structure of disparlure indicates that an immediate direct precursor is the alkene 2me-7-18:Hc. In fact, identification of the alkene aided in identifying disparlure (14), and there is some indication that the alkene can be converted to disparlure (15). Therefore, we started by determining how the alkene was produced, specifically the precursor for the methyl branch. Methyl-branched hydrocarbons are biosynthesized by using specific precursors depending on where the methyl branch is located in the final product (16). Propionate is used during chain elongation to introduce internal methyl branches (17). Leucine and valine contribute the carbons for methyl groups on the number two carbon of odd and even chain-length hydrocarbons, respectively (16, 18). Therefore, we started by determining whether valine is incorporated into the even-chain alkene.

D_8-valine was injected once daily for 3 days starting with 1-day-old females. One hour after the last injection, hemolymph was collected and analyzed for incorporation into the alkene (Fig. 2*A*). The D_8-valine injection results clearly show that seven deuteriums were found in the alkene. Note that one deuterium is lost during the conversion of valine to isobutyryl-CoA. The intensities of ions 50 and 273 indicate deuterium labeling. These peaks elute slightly ahead of the unlabeled endogenous compound, which is indicative of deuterium-containing compounds. Valine also labeled the saturated 2me-C18 that was found in the hemolymph, as indicated by the second ion 50 peak. We also analyzed disparlure extracted from pheromone glands (Fig. 2*B*) and found label incorporation. Deuterium incorporation was shown by the intensities of ions 50 and 148. Ion 190 was also monitored, but a peak was not detected, indicating that the deuteriums were not located in that fragment. The ion 148 peak indicates that the deuterium atoms are located on the methyl-branch side of the molecule. We also examined the alkene obtained from D_8-valine injections after making dimethyl–disulfide derivatives (11). The deuterium labeling was found on the methyl side of the double bond (data not shown). Females were also injected with unlabeled valine and subjected to the same procedures. Peaks were not found corresponding to the deuterium-labeled peaks in females injected with D_8-valine (data not shown). These data indicate that valine contributes the carbons for the methyl group. Valine is converted to isobutyryl-CoA, which is used for chain initiation of the hydrocarbon.

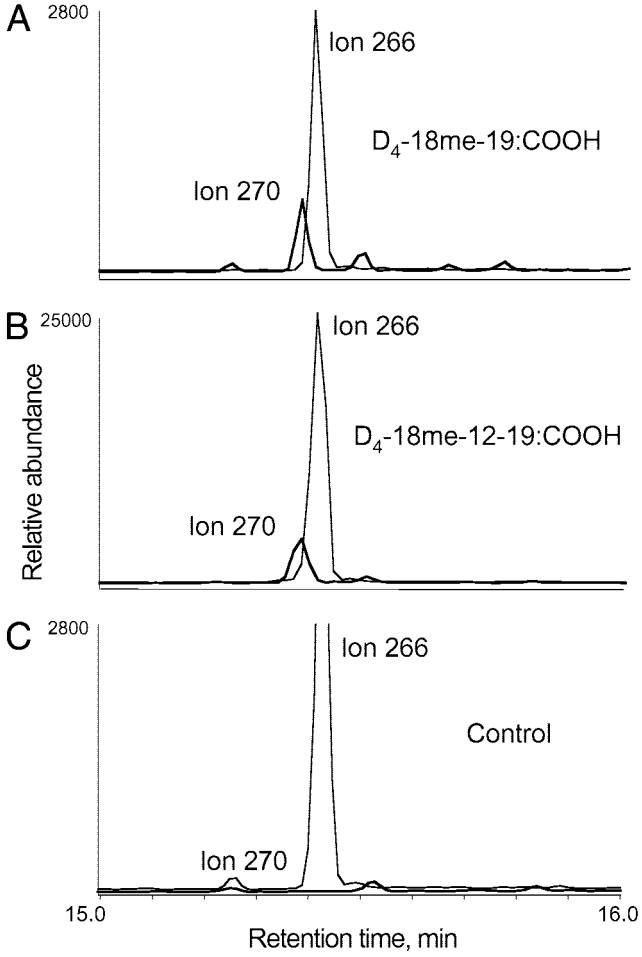

Fig. 3. Incorporation of D_4-18me-19:COOH (*A*) and D_4-18me-12-19:COOH (*B*) into the alkene, 2me-7-18:Hc. Isolated cuticular abdominal tissue was incubated with the deuterium-labeled compound (25 μg per 100 μl of Grace's medium) for 6 h, and then the tissue was extracted and hydrocarbons were purified and analyzed by GC/MS. MS was performed in the SIM mode, monitoring for the molecular ion 266 and ion 270 for label incorporation. (*C*) Control tissues were incubated with Grace's medium without labeled compounds.

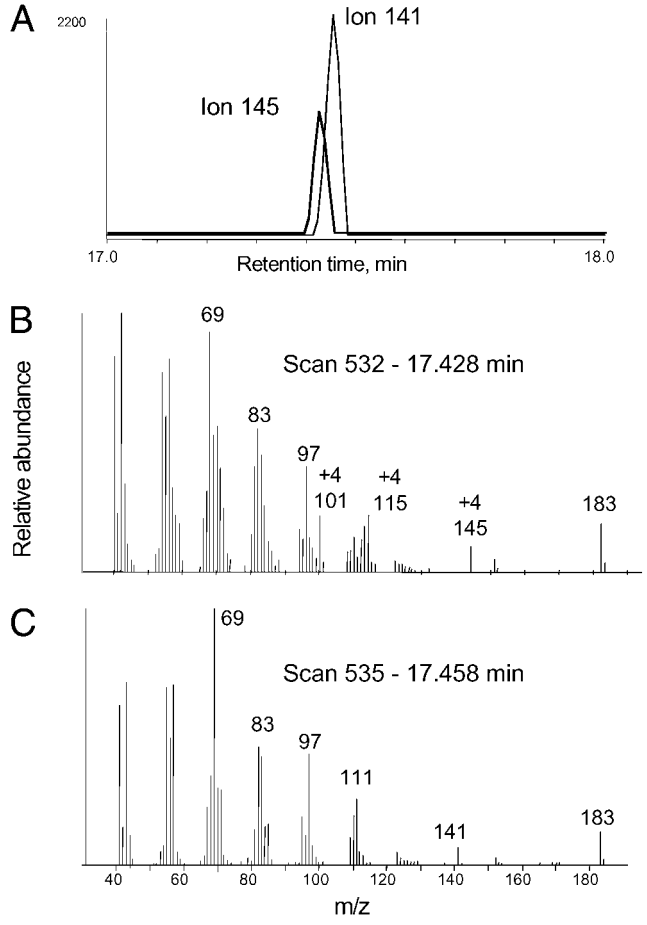

Fig. 4. Incorporation of D_4-2me-7-18:Hc into disparlure after injection into females. One-day-old females were injected with 5 μg of D_4-2me-7-18:Hc in 5 μl of Grace's medium once per day for 3 days. Two hours after the last injection, pheromone glands were removed, extracted, and analyzed for incorporation by complete scan GC/MS. (*A*) Ions 141 and 145 were selected from the scan for presentation. (*B*) Complete ion scan under the ion 145 peak. (*C*) Complete ion scan under the ion 141 peak.

Hydrocarbon chain elongation is thought to occur with a microsomal fatty acid synthetase (19). The alkene would be produced by chain elongation to 19 carbons yielding 18me-19:CoA. The double bond would then be introduced by using a desaturase, likely a Δ12 desaturase that introduces the double bond 12 carbons from the carboxyl end. The resulting unsaturated fatty acid would then be decarboxylated to the alkene. To determine these intermediate steps in the biosynthetic pathway, deuterium-labeled 18me-19:COOH and 18me-12-19:COOH were synthesized and incubated with isolated abdominal tissue. As indicated in Fig. 3, both were incorporated into the alkene. The molecular ion 266 was monitored plus ion 270, because the labeled intermediates each had four deuterium atoms located on carbons 5 and 6 from the methyl end. Control tissues did not exhibit a corresponding ion 270 peak.

These results indicate that a Δ12 desaturase most likely introduces the double bond into the alkene. A variety of desaturases have been implicated in sex pheromone biosynthesis in moths, but this one is unusual in that it uses a methyl-branched saturated fatty acid as a substrate. Two Δ12 desaturases have been previously identified in insects; however, both use a monounsaturated fatty acid as a substrate. One uses Z9-14:COOH to make Z9,E12-14:COOH, which is then converted to Z9,E12-14:OAc, the sex pheromone of several moths (20). The other is found not in moths but in several other insects and uses oleic acid (Z9-18:COOH) to make linoleic acid (Z9,Z12-18:COOH) (21). Future research may yield comparisons of these unique desaturases, similar to those being made with other moth desaturases (22, 23).

Unsaturated methyl-branched hydrocarbons are not used by many species of moths (H. Arn, www.nysaes.cornell.edu/pheronet/ or www-pherolist.slu.se/). *Scoliopteryx libatrix* uses 13me-Z6-21:Hc (24), and *Lyonetia clerkella* uses 14me-1-18Hy (25). In addition, *Lymantria monacha* uses 2me-Z7-18:Hc as part of its sex pheromone blend as well as Z7-18:Hc (26). The biosynthesis of the latter compound probably occurs along a similar pathway as 2me-Z7-18:Hc, except propionyl-CoA is used as the starting substrate for the fatty acid synthetase.

Hydrocarbon biosynthesis occurs in specialized cells called oenocytes, which can be associated with epidermal cells in the abdomen (27). The lipid protein carrier lipophorin then transports the newly synthesized hydrocarbon from the oenocytes to epidermal cells throughout the insect where the hydrocarbon is transferred to the cuticular surface (28). In the arctiid moth, *Holomelina aurantiaca*, the transport of pheromone is very specific in that only the pheromone is unloaded at the phero-

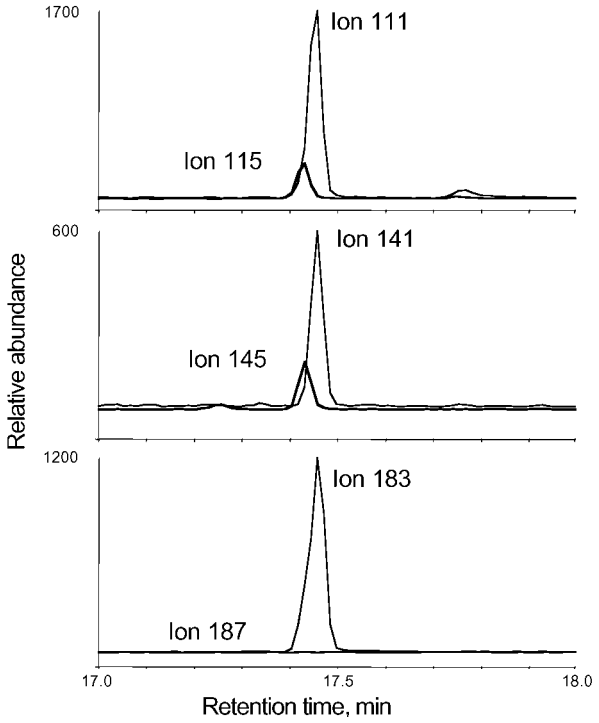

Fig. 5. Incorporation of D_4-2me-7-18:Hc into disparlure in isolated pheromone glands. Pheromone glands were incubated with 2.5 μg of D_4-2me-7-18:Hc in 5 μl of Grace's medium for 3 h; then the glands were extracted and analyzed by SIM GC/MS.

mone gland, whereas hydrocarbon destined for the cuticular surface is not (29). To determine where in the gypsy moth female the alkene is produced, isolated tissues were incubated with D_8-valine. Incorporation occurred only in the isolated abdomen with attached epidermal and oenocyte cells (data not shown). Hemolymph also was shown to contain the alkene but not disparlure (11), indicating that the alkene is biosynthesized in oenocyte cells and released into the hemolymph.

To demonstrate the conversion of the alkene to disparlure, deuterium-labeled alkene, D_4-2me-7-18:Hc, was synthesized and injected into 1-day-old females. Two hours after the last injection, pheromone glands were removed and analyzed for incorporation by total ion scan GC/MS. As shown in Fig. 4, label was incorporated into disparlure. Fig. 4*A* shows ion 141, which comes from the unlabeled endogenous disparlure, and ion 145, which comes from the labeled disparlure. Fig. 4 *B* and *C* display the total ion scan for the labeled and endogenous disparlure, respectively. The ions at 101, 115, and 145 indicate deuterium labeling with an absence of ion 187 (Fig. 4*B*), indicating that the deuteriums were found on the methyl-branched side of the epoxide.

To determine where the conversion to disparlure takes place, isolated tissues were incubated with D_4-2me-7-18:Hc. Isolated pheromone glands were incubated with D_4-2me-7-18:Hc in Grace's medium for 3 h and then extracted and analyzed for incorporation by SIM GC/MS. Conversion to disparlure took place only in isolated pheromone glands (Fig. 5). Ions 115 and 145 indicate deuterium labeling of disparlure. The lack of an ion 187 indicates correct positional labeling of the deuterium atoms. These results indicate that the alkene is released into the hemolymph, where it is transported to the pheromone gland for conversion to disparlure. Once the conversion is made, the sex pheromone is released into the environment.

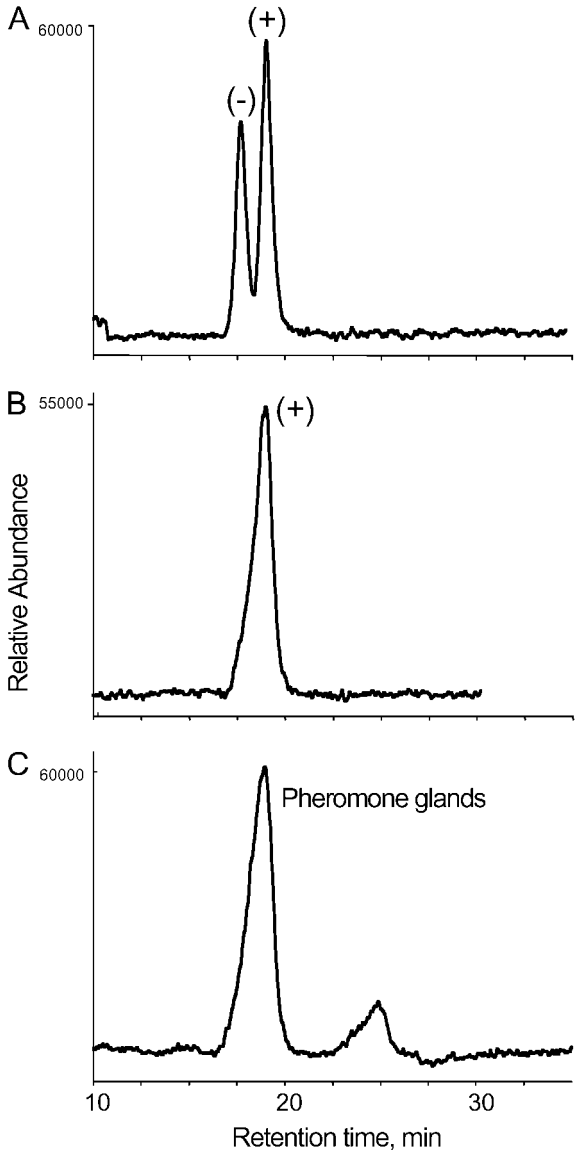

Fig. 6. Partial HPLC/MS chromatograms of racemic mixture of disparlure (*A*), (+)-disparlure (*B*), and disparlure isolated from four pheromone glands (*C*). HPLC/MS conditions are described in *Materials and Methods*. The chromatograms represent SIM of ion 300 (M + 18).

It is thought that female gypsy moths produce primarily the (+)-isomer of disparlure, which was demonstrated by using stereo-pure pheromone in field and laboratory bioassays (10, 30, 31). However, male gypsy moths can discriminate between the two isomers, with (+)-disparlure being stimulatory and (–)-disparlure being inhibitory. In contrast, the nun moth, *L. monacha*, responds only to the (+)-isomer, with the (–)-isomer having no effect on attraction (8), although the female produces other compounds that are needed for male nun moth attraction (26). It is thought that the female nun moth produces ≈90% (–)-disparlure and 10% (+)-disparlure, and the gypsy moth, 100% (+)-disparlure (8). These ratios were deduced from studies using wind tunnel and electroantennogram procedures. To determine whether the gypsy moth is producing the (+)-isomer, we used chiral HPLC coupled with MS detection. Chiral separation of disparlure obtained from pheromone glands indicates that primarily the (+)-isomer is present (Fig. 6). Because baseline separation of the isomers did not occur, we cannot rule

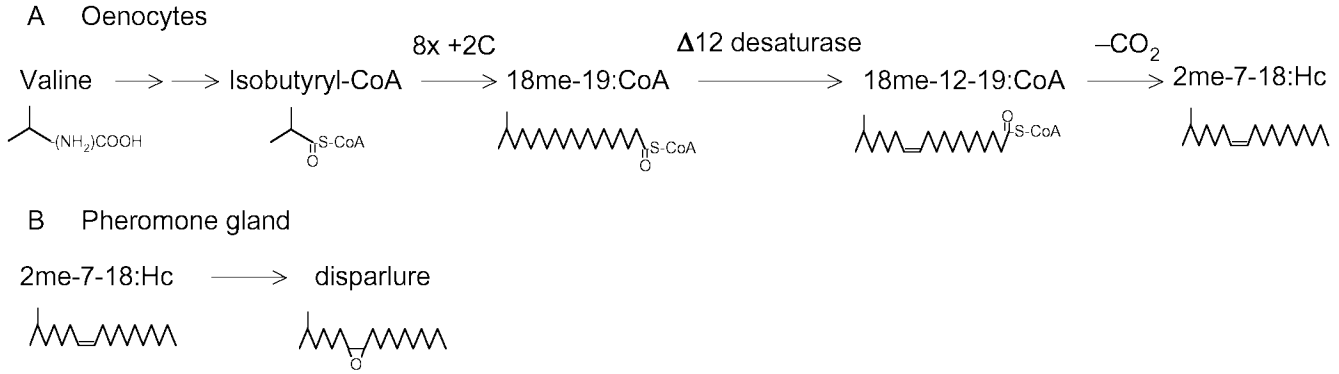

Fig. 7. Proposed biosynthetic pathway for producing the alkene (*A*) and disparlure (*B*) by oenocyte and pheromone gland cells, respectively.

out the possibility that some (–)-isomer is present. However, both the behavioral and chromatographic data indicate that the female gypsy moth produces the (+)-isomer. Comparative information may be obtained with the nun moth, which should make primarily the (–)-isomer.

These results taken together indicate a biosynthetic pathway outlined in Fig. 7, where valine contributes the carbon for the methyl-branched group and chain initiation. Chain elongation continues to 19 carbons, and then a Δ12 desaturase introduces the double bond. Decarboxylation produces the alkene, which is then transported to the pheromone gland for conversion to (+)-disparlure.

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